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ORIGINAL ARTICLE

Quantitative *Fusarium* spp. and *Microdochium* spp. PCR assays to evaluate seed treatments for the control of *Fusarium* seedling blight of wheat

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Fusarium seedling blight, internal transcribed spacer, *Microdochium* spp., quantitative PCR, seed-borne disease.

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Abstract

Aims: To develop sensitive quantitative PCR assays for the two groups of pathogens responsible for *Fusarium* seedling blight in wheat: *Fusarium* group (*Fusarium culmorum* and *Fusarium graminearum*) and *Microdochium* group (*Microdochium nivale* and *Microdochium majus*); and to use the assays to assess performance of fungicide seed treatments against each group.

Methods and Results: Primers conserved between the species within each group were used to develop competitive PCR assays and used to quantify DNA of each group in wheat seed produced from inoculated field plots. Seed was used in seed treatment efficacy field experiments and the amount of DNA of each group was determined in emerged seedlings. The performance of treatments towards each group of pathogens was evaluated by comparison of the reduction in DNA in seedlings emerged from treated seed compared with untreated seed.

Conclusions: DNA from the two groups of pathogens causing *Fusarium* seedling blight of wheat can be quantified separately using the competitive PCR assays. These assays show improved sensitivity compared with those previously reported for the individual species and allowed the quantification of pathogen DNA in seed and seedlings. Significant reductions in pathogen DNA were evident for each seed treatment.

Significance and Impact of the Study: Quantification of DNA for each group allows the evaluation of seed treatment performance towards the two components of *Fusarium* seedling blight disease complex. The approach taken and the assays developed in this study will be of use for the study of other *Fusarium* disease complexes and their control. Based on the results reported here on the seedling stage of crop development, further studies that examine the control of seed-borne pathogens through fungicide seed treatments throughout the growing season are warranted.

Introduction

Fusarium seedling blight can result in the death of seedlings before or just after seedling emergence (Wiese 1987). Those plants that survive infections at the seedling stage are likely to exhibit symptoms of *Fusarium* foot rot later in the growing season, the severity of which is affected by environmental conditions (Colhoun 1970; Cook 1980). Although many species of *Fusarium* have been isolated from wheat, the major pathogens of this disease complex are considered to be *Fusarium avenaceum*, *Fusarium culmorum*, *Fusarium graminearum* and *Fusarium*

poae (Parry *et al.* 1995; Doohan *et al.* 2003). Studies have shown that *F. culmorum* and *F. graminearum* are the most pathogenic species, whereas *F. poae* and *F. avenaceum* are weakly pathogenic (Colhoun *et al.* 1968; Browne and Cooke 2005; Fernandez and Chen 2005). *Fusarium* seedling blight can also be caused by the *Microdochium* spp. pathogens *Microdochium nivale* (Fr.) Samuels and I. C. Hallett and *Microdochium majus* (Wollenw.) Glynn and S.G. Edwards, comb. nov. In the cooler regions of northern Europe, *M. nivale* and *M. majus* are often the predominant pathogens (Daamen *et al.* 1991) whereas in warmer regions, *F. culmorum* and *F. graminearum* are usually the dominant species (Parry *et al.* 1995). Seedling blight infections generally arise from seed-borne inoculum (Colhoun 1970). Soil-borne inoculum rarely causes seedling blight although soil-borne *Fusarium* can damage seedlings in warm, arid soils; *M. nivale* and *M. majus* which are less mobile in soil favour cool, dry soils (Millar and Colhoun 1969). Little data exist on the frequency of *Fusarium* spp. present in UK wheat seed, however, *F. culmorum* and *F. graminearum* represented 28% and 69% of 299 *Fusarium* isolates examined in Dutch wheat seed in 2000 and 2001, *F. poae* and *F. avenaceum* made up the remaining 3% of isolates (Waalwijk *et al.* 2003).

Fungicide seed treatment is the primary control measure for eradicating the seed-borne inoculum leading to *Fusarium* seedling blight. The performance of fungicides towards pathogens in disease complexes such as *Fusarium* seedling blight, foot rot and head blight are often difficult to measure. The interpretation of visual disease assessments in field trial experiments is complicated by the significant presence of endogenous disease causing fungi. Visual symptoms on emerged seedlings cannot be used to assess the performance of seed treatments towards individual species unless known species are present alone; this is difficult to achieve under field experiment conditions. Further, the early stages of infection by *Fusarium* disease-causing pathogens are often symptomless (Parry *et al.* 1994). Isolation of *Fusarium* disease-causing pathogens in axenic culture can provide an indication of the incidence of the fungal species involved. However, such isolations cannot quantify the degree of infection or the potential of the infective agent(s) to cause foot rot later in the season (Hare 1997), isolation techniques also favour those species which grow quickest under the culture conditions employed.

PCR can be used to identify pathogens at any taxonomic level. It is useful to detect or quantify the principal fungal taxa or group responsible for disease (Simpson *et al.* 2001) rather than the individual species which could be present. Competitive PCR allows the determination of the amount of pathogen DNA in infected samples. Relative fungal DNA concentration in plant material has been shown to be a good indicator of future seedling blight disease development (Glynn 2002). In addition, a positive correlation was found between the amount of *Microdochium* spp. DNA and the number of infected seeds (Cockerell *et al.* 2004). Competitive PCR has previously been used to study the epidemiology and control of several *Fusarium* disease complex pathogens of wheat (Doohan *et al.* 1999; Edwards *et al.* 2001; Nicholson *et al.* 2002).

The method traditionally employed to determine the severity to which individual seed batches or seedlings are infected is to determine the percentage of seeds or stems from which pathogen colonies emanate following surface sterilization and incubation on appropriate media. Although these methods can detect symptomless infection and identify the species present they are unable to determine the amount of pathogen. In the case of seedlings, visual symptoms can also be used to determine the severity of infection, however visual symptoms are unable to differentiate between pathogens.

In this study, we considered the pathogens responsible for *Fusarium* seedling blight of wheat as being from two groups, namely; *Fusarium* group (*F. culmorum* and *F. graminearum*) and *Microdochium* group (*M. nivale* and *M. majus*) as each group favours separate environmental conditions for disease and have been shown to have differing responses to fungicides (Pettitt *et al.* 1993; Simpson *et al.* 2001). Quantification of DNA from the two groups, allows the performance of seed treatments to be determined more efficiently. It is desirable to detect these pathogens in material containing a low amount of inoculum such as in infected seed and in the early stages of seedling infection. Beck (1998) designed a range of genus- and species-specific PCR primer pairs for various plant pathogenic genera and species based on sequences within the internal transcribed spacer (ITS) regions of ribosomal DNA. The numerous identical copies of ribosomal DNA genes increases the sensitivity of the PCR assay over single copy genes. These primers allowed the development of highly sensitive PCR assays specific to a number of important plant pathogenic species and genera which included primers for *Fusarium* (specifically *F. culmorum* and *F. graminearum*) and *Microdochium* (specifically *M. nivale* and *M. majus*) (Beck 1998). Quantitative PCR has the advantage over diagnostic PCR of allowing the amount of a particular pathogen present in infected plant material to be determined.

Aim

(i) To develop a sensitive, quantitative PCR assay to determine the amount of fungal DNA in plant material for the two groups of pathogens responsible for *Fusarium* seedling blight in wheat: (a) *F. culmorum* and *F. gramine-*

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arum and (b) *M. nivale* and *M. majus*; (ii) to assess the performance of fungicide seed treatments against each group using PCR assays.

Materials and methods

Origin of fungal isolates

Fungal strains used in this study and their sources are listed in Table 1.

Source of seeds and seedlings

Standard crop husbandry practices were used to maintain 12 plots (10 × 2 m) of wheat cv. Hussar (here on referred to as experiment 1) and cv. Equinox (here on referred to as experiment 2). Four plots were inoculated at mid-anthesis (growth stage 65) (GS 65) (Zadoks *et al.* 1974) with a conidial suspension from five isolates (2×10^5 spores per ml) of a seedling blight pathogen at a rate of 33 ml m^{-2} using a knapsack sprayer. In experiment 1, plots were inoculated with *F. culmorum*, *F. graminearum* or *M. majus*; in experiment 2, plots were inoculated with *F. culmorum*, *M. majus* or *M. nivale*. Plots were mist irrigated for 21 days as previously described Hilton *et al.* 1999) to aid infection. Four uninoculated, non-misted plots were used as guards between each set of inoculated misted plots. Grain was harvested at GS 92 using a Seedmaster Plot combine (Wintersteiger, Austria).

Four seed lots in each experiment 1–4 (experiment 1), 5–8 (experiment 2) were used in fungicide seed treatment efficacy experiments. Seed lots 1 and 5 were commercial seed, seed lots 2 and 6 were from field plots inoculated with *F. culmorum*, seed lot 3 *F. graminearum*, seed lots 4 and 7 *M. majus* and seed lot 8 *M. nivale*. Seed was treated with either Beret Gold (Syngenta) (a.i. fludioxonil: 24·3 g l^{-1}) or Sibutol (Bayer) (a.i. bitertanol + fuberidazole: 375 + 23 g l^{-1}) at the label recommended rate, untreated seed was used as a control. In both experiments 1 and 2, seed was drilled according to a randomized block design with four replicates at Edgmond (Shropshire, UK). At GS 13, 30 seedlings were removed from each plot, the roots, remaining seed coat and any soil debris were removed and the seedlings were cut to 4 cm in

Table 1 Source of fungal isolates and reaction obtained using JBF and JBM primers

Isolate	Species	Origin	PCR product*	
			JBF primers	JBM primers
95w	*Fusarium culmorum*	England	+	–
017	*F. culmorum*	England	+	–
302	*F. culmorum*	England	+	–
421/5	*F. culmorum*	France	+	–
421/14	*F. culmorum*	France	+	–
405/11	*F. culmorum*	Germany	+	–
NFTP	*Fusarium graminearum*	England	+	–
113	*F. graminearum*	England	+	–
145	*F. graminearum*	England	+	–
86	*F. graminearum*	France	+	–
507	*F. graminearum*	France	+	–
405/1	*F. graminearum*	Germany	+	–
74/1/N	*Microdochium nivale*	England	–	+
2a	*M. nivale*	UK	–	+
SWG052/1/N	*M. nivale*	Germany	–	+
NZ013/5/N	*M. nivale*	New Zealand	–	+
JP101046[d]	*M. nivale*	Japan	–	+
NL005/1/N	*M. nivale*	the Netherlands	–	+
24/3/M	*Microdochium majus*	England	–	+
NL007/1/M	*M. majus*	the Netherlands	–	+
F060/1/M	*M. majus*	France	–	+
NZ25/GVR/M	*M. majus*	New Zealand	–	+
JP236880†	*M. majus*	Japan	–	+
47b	*M. majus*	Holland	–	+

Isolates in bold were used in the quantitative PCR validation.

*+, a positive PCR product using PCR primer combination JBF or JBM; –, no PCR product was present.

†Isolates were from the National Institute of Agrobiological Resources (Kannondai, Japan) and others were from the Harper Adams University College (Shropshire, UK).

length from the base of the stem, washed, placed in plastic kartell tubes (Fisher, Loughborough, UK) and freeze dried prior to DNA extraction.

DNA extraction

The fungi were grown in 150 ml potato dextrose broth inoculated with mycelial fragments from potato dextrose agar cultures. Cultures were incubated in an orbital incubator at 28°C, 100 rev min^{-1} for 7–11 days. Fungal mycelia were pelleted by centrifugation and ground in liquid nitrogen. Total genomic DNA was extracted from the ground mycelia using the protocol of Lee and Taylor (1990). DNA for fungal standards used in quantitative PCR reactions was extracted from 7-day-old cultures and diluted to 1 ng μl^{-1} in Tris–EDTA (TE) buffer according to the methods described previously (Glynn *et al.* 2005).

DNA extracted from seed was performed as described in Edwards *et al.* (2001). Extraction of DNA from seedlings was performed using a modified method of Edwards *et al.* (2001) described for seed material. Seedlings were freeze dried and then milled to a fine powder by adding three sterile 8-mm diameter steel ball bearings and shaken for 1 h using a soil mill (Griffin, London, UK). Plant material was transferred to a sterile 50-ml centrifuge tube and 10 ml of cetyl trimethyl ammonium bromide extraction buffer was added. Tubes were incubated at 65°C for 1 h, cooled to 20°C and 3·3 ml of potassium acetate (5 mol l^{-1}) was added. Total DNA was purified, quantified and diluted to 40 ng μl^{-1}.

Development of quantitative PCR assays for *Fusarium* and *Microdochium* seedling blight pathogen DNA

Oligonucleotide primers designed on regions of rDNA for *Fusarium* seedling blight pathogens; *F. culmorum* and *F. graminearum* (JB566, 5′-GTTTTTAGTGGAACTTCTGAG-T-3′ and JB572, 5′-AAGTTGGGGTTTAACGGC-3′ here on referred to as JBF) and *Microdochium* seedling blight pathogens; *M. nivale* and *M. majus* (JB612, 5′-GGTGC-TGTCTCTCGGGAC-3′ and ITS4, 5′-TCCTCCGCTTATT-GATATGC-3′ here on referred to as JBM) as described previously (Beck 1998) were used. DNA from all isolates detailed in Table 1 was amplified with the JBM and JBF assays. Diagnostic PCR amplifications were carried out using JBM or JBF primers in total reaction volumes of 25 μl containing 100 μmol l^{-1} of each nucleotide, 100 nmol l^{-1} of each forward and reverse primer, 20 units ml^{-1} of Super *Taq* polymerase (Kramel Biotech, Cramlington, UK), 10 mmol l^{-1} Tris–HCl, 1·5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl, 0·1 mg ml^{-1} of gelatin (Sigma), 0·5 mg ml^{-1} of Tween 20 (Sigma) and 0·5 mg ml^{-1} of Nonidet P-40 (Sigma) and 5 μl of sample DNA. Positive controls contained either 5 μl of *M. nivale* or *F. culmorum* DNA (1 ng μl^{-1}) instead of sample DNA and negative controls contained 5 μl of water. Thermocycling consisted of an initial denaturation step of 95°C for 75 s followed by 35 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 45 s and a final extension step of 72°C for 4 min and 15 s. Electrophoresis was performed using 2% agarose gels containing 0·5 μg of ethidium bromide per ml in TAE buffer (40 mmol l^{-1} Tris-acetate, 1 mmol l^{-1} EDTA, pH 8·0), amplified products were observed under UV light.

Internal standards (JBMIS and JBFIS) were constructed for competitive PCR assays based on the method of Edwards *et al.* (2001) from a 1·2-kb fragment of the onion (*Allium cepa*) gene alliinase (EMBL accession code L48614). The fragment was used as a template and amplified using primers ONI/F (5′-TGCTCTGCTGATG-TTGCCAG-3′) and ONI/R (5′-TACATGGGGATGGA-GGTCTC-3′). Reaction conditions, thermocycling and electrophoresis were as described above with the exception that an annealing temperature of 58°C was used. The amplicon was excised from the gel following electrophoresis, placed in 1 ml of TE buffer and incubated at 4°C for 16 h. A 5-μl aliquot served as template DNA in a PCR reaction with the linker primers (*Microdochium*: MNIV/FL (5′-CTCTCGGGACGTTGCTCATGCCCC-3′), NUC4/RL (5′-ATTGATATGCTCTCGGGAAGTGCC-3′) or *Fusarium*: JBF3/FL (5′-ACTTCTGAGTAGGAAATGC-AGCGG-3′), JBF4/RL (5′-GTTTAACGGCTGAGGTCGC-GCATG-3′). The concentrations of reaction ingredients were as described above, the thermocycling programme used consisted of 10 cycles with an annealing temperature of 38°C followed by 20 cycles with an annealing temperature of 55°C. Amplified fragments (628 bp *Microdochium* and 534 bp *Fusarium*) here on referred to as JBM and JBF internal standard DNA (JBMIS and JBFIS) were cloned using the pGEM®-T vector system (Promega, Southampton, UK) according to the standard protocol suggested by the manufacturer.

Quantitative PCR

Internal standard DNA (JBMIS and JBFIS) and DNA from *M. nivale* and *F. culmorum* were diluted and amplified together with JBM or JBF primers to determine the concentration giving the greatest sensitivity and dynamic range. Stock internal standard DNA (2·9 fg μl^{-1} JBMIS; 38·3 fg μl^{-1} JBFIS) was prepared in TE buffer in the presence of 10 ng μl^{-1} carrier (herring sperm) DNA to improve stability during storage at −20°C. A standard curve was generated for each primer pair, *M. nivale* DNA was diluted two-fold over the range 0·003–1·6 pg μl^{-1} for use with JBM primers. *Fusarium culmorum* DNA was

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diluted twofold over the range of 0·0008–6·4 pg μl^{-1} for use with JBF primers. Ten microlitres of each dilution was amplified in the presence of 10 μl of the respective internal standard in a final volume of 50 μl. PCR reaction conditions were as described previously for the diagnostic PCR protocol. To quantify the amount of *Microdochium* or *Fusarium* DNA in total DNA extracted from seed and seedlings, 50 μl of competitive PCR reactions were setup each containing 10 μl of sample DNA and 10 μl of JBMIS or JBMIS respectively. The concentrations of reaction ingredients and thermocycling conditions were as described above.

Gels were viewed under UV light on a Gel Doc 1000 fluorescent gel documentation system (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) following electrophoresis. Unsaturated images were analysed using Molecular Analyst software (Bio-Rad). PCR product ratios were determined for each standard and sample by dividing the band intensity of the JBM or JBF product (472 or 346 bp) by that of the JBMIS or JBFIS product (628 or 534 bp). Quantification of amplified fungal DNA was determined by comparison of sample ratio to the respective standard curve and was expressed as concentration of fungal DNA as a proportion of total DNA.

Quantitative PCR validation

To validate that each species was amplified with equal efficiency within the quantitative PCR, DNA from three isolates of *F. culmorum*, *F. graminearum*, *M. nivale* and *M. majus* (detailed in Table 1) was extracted and diluted to 1 pg μl^{-1} and quantified using the corresponding assays detailed above. Isolates were amplified in quadruplicate to provide adequate degrees of freedom for analysis.

Statistical analysis

Results were analysed by ANOVA using Genstat 5·4.1 (Lawes Agricultural Trust, Harpenden, UK). Where necessary, data were transformed to obtain normal distributions. Individual treatments were compared using the least significant difference at the 5% significance level. All statistical significance quoted is at the 5% level unless stated otherwise.

Results

Diagnostic and quantitative PCR validation

All isolates tested amplified with the correct primer pair and no misamplification was detected (Table 1). ANOVA of the DNA concentration of the three isolates of *M. nivale* and *M. majus* indicated that there was no difference in the amplification of the two species using the JBM assay ($P = 0{\cdot}80$). The average concentration of the 1 pg μl^{-1} stocks as determined by the JBM PCR assay was 1·26 pg μl^{-1}. ANOVA of the DNA concentration of the three isolates of *F. culmorum* and *F. graminearum* indicated that there was no difference in the amplification of the two species using the JBF assay ($P = 0{\cdot}86$). The average concentration of the 1 pg μl^{-1} stocks as determined by the JBF PCR assay was 1·06 pg μl^{-1}.

Quantification of pathogens in seed

Pathogen DNA from each *Fusarium* seedling blight group was detected and quantified in all eight seed lots. The commercial seed lots in both experiments (lots 1 and 5) had the lowest amount of *Microdochium* group DNA (Fig. 1). In experiment 1, the commercial seed lot (lot 1) had the lowest amount of *Fusarium* group DNA and in experiment 2, it contained the second lowest amount of *Fusarium* group pathogen (lot 5). Seed produced from field plots inoculated with either a *Microdochium* or a *Fusarium* species contained high amounts of *Microdoch-*

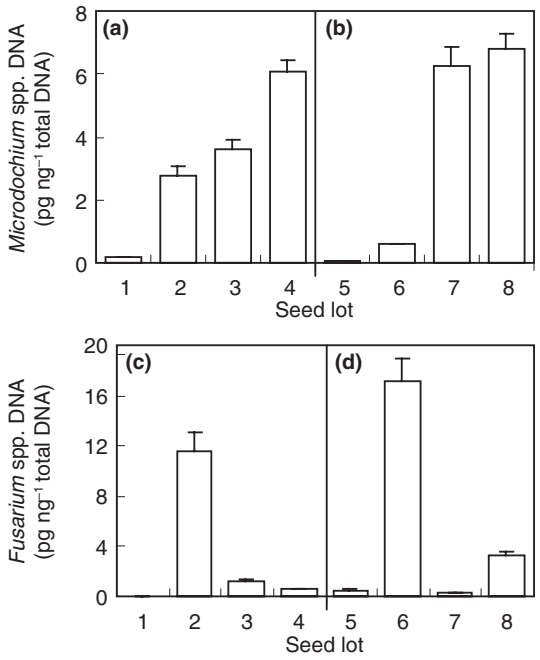

Figure 1 Quantification of *Microdochium* seedling blight pathogen DNA in seed lots for: (a) experiment 1 (cv. Hussar) seed and (b) experiment 2 (cv. Equinox) seed; *Fusarium* seedling blight pathogen DNA in seed lots for: (c) experiment 1 seed and (d) experiment 2 seed. Key to seed lots: 1 and 5, commercial seed; 2 and 6, from field plots inoculated with *Fusarium culmorum*; lot 3, *Fusarium graminearum*; lots 4 and 7, *Microdochium majus*; lot 8, *Microdochium nivale*. Bars indicate standard errors of the mean values.

ium or *Fusarium* group DNA, respectively, with the exception of the *F. graminearum* inoculated plots. The amount of *Microdochium* DNA was significantly different between all experiment 1 seed lots, ranging from 0·19 pg ng^{-1} total DNA in seed lot 1 to 6·1 pg ng^{-1} total DNA in seed lot 4 (Fig. 1a). With the exception of seed lots 7 (5·9 pg ng^{-1} total DNA) and 8 (6·8 pg ng^{-1} total DNA) which contained the most, the amount of *Microdochium* DNA in each experiment 2 seed lot was significantly different (Fig. 1b). Quantification of *Fusarium* DNA showed significant differences between all seed lots in experiment 1, seed lot 2 contained the most (17 pg ng^{-1} total DNA) followed by seed lots 3, 4 and 1 (Fig. 1c). Seed lot 6 contained the most *Fusarium* DNA in experiment 2, significantly more than seed lot 8. No significant difference was observed in the amount of *Fusarium* group DNA in seed lots 5 and 7 although they did contain significantly less *Fusarium* DNA than seed lot 8 (Fig. 1d).

Quantification of *Microdochium* seedling blight pathogen DNA in seedlings

Microdochium seedling blight pathogen DNA was detected in all seedling samples in both experiments (Fig. 2a,b). Seedlings produced from commercial seed (lots 1 and 5) contained significantly less fungal DNA than seedlings from untreated seed from the three infected seed lots in each experiment. In experiment 1, no significant difference was observed between *Microdochium* DNA in seedlings produced from untreated seed from seed lots 3 and 4 although this was significantly more than from seed lot 2. In experiment 2, no significant difference was observed in the amount of *Microdochium* DNA detected in seedlings from untreated seed from lots 6 and 7 and, this was less than that from untreated seed lot 8. In experiment 1, the quantity of *Microdochium* DNA was reduced significantly using bitertanol + fuberidazole for seedlings from seed lots 2, 3 and 4 (98%, 70% and 92% reduction respectively) although not for seed lot 1 which had the lowest amount of *Microdochium* infection (19% reduction). Treatment with fludioxonil resulted in significant reductions in *Microdochium* DNA in seedlings from seed lots 2, 3 and 4 (99·5%, 87% and 93% reduction respectively) although not for seed lot 1 (48% reduction). In experiment 2, the quantity of *Microdochium* DNA was reduced significantly in seedlings from all seed lots treated with either bitertanol + fuberidazole (seed lot 5: 99·6% reduction, seed lot 6: 84·5% reduction, seed lot 7: 69·7% reduction and seed lot 8: 93% reduction) or fludioxonil (seed lot 5: 95% reduction, seed lot 6: 99·9% reduction, seed lot 7: 99·5% reduction and seed lot 8: 99·9% reduction). Fludioxonil showed a significant reduction in *Microdochium* DNA compared with bitertanol + fuberidazole in seedlings produced from seed lot 7.

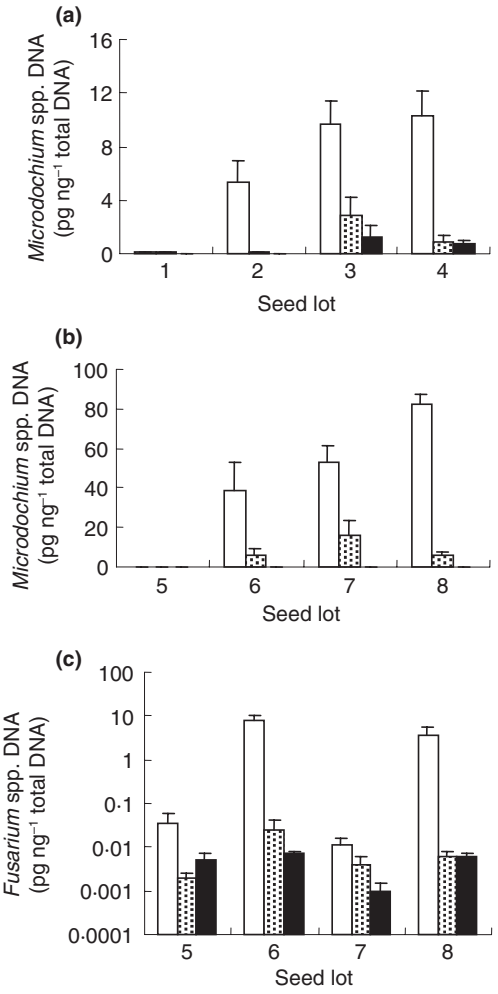

Figure 2 Quantification of *Microdochium* seedling blight pathogen DNA in experiment 1 (cv. Hussar) (a) and experiment 2 (cv. Equinox) (b), and *Fusarium* seedling blight pathogen DNA in experiment 2 (cv. Equinox) (c) in seedlings from four seed lots treated with (■) fludioxonil (48·6 g ai/100 kg seed), (▨) bitertanol + fuberidazole (375 g ai + 23 g ai/100 kg seed) and (□) untreated control. Key to seed lots: 1 and 5, commercial seed; 2 and 6, from field plots inoculated with *Fusarium culmorum*; lot 3, *Fusarium graminearum*; lots 4 and 7, *Microdochium majus*; lot 8, *Microdochium nivale*. Bars indicate standard errors of the mean values.

Quantification of *Fusarium* seedling blight pathogen DNA in seedlings

In experiment 1, *Fusarium* seedling blight pathogen DNA was only detected in seedlings produced from untreated seed lot 2 (34·0 pg ng^{-1}). In experiment 2, *Fusarium* DNA was detected in all samples (Fig. 2c). No significant difference was observed in the amount of *Fusarium* DNA detec-

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ted in untreated seedlings from seed lots 6 and 8 and these samples contained significantly more *Fusarium* DNA than seedlings from untreated seed lots 5 and 7. *Fusarium* DNA was reduced significantly for all four seed lots using bitertanol + fuberidazole (seed lot 5: 94% reduction, seed lot 6: 99·7% reduction, seed lot 7: 64% reduction and seed lot 8: 89% reduction) or fludioxonil (seed lot 5: 85% reduction, seed lot 6: 99·9% reduction, seed lot 7: 91% reduction and seed lot 8: 94% reduction). No significant difference was observed between seed treatments.

Discussion

Numerous molecular systematic studies have utilized the coding and non-coding sequence of rDNA to distinguish fungal species. Areas of rDNA that were highly conserved between many species of filamentous fungi have been reported (White *et al.* 1990) and primers designed in these regions have proved useful for taxonomic studies within *Fusarium* (Bateman *et al.* 1996; Waalwijk *et al.* 1996). In the present study, primers based on rDNA were used to separately detect the two groups of *Fusarium* disease-causing pathogens in wheat. Ribosomal genes represent good targets for PCR assay development due to their high copy number. Previous quantitative PCR assays for the individual *Fusarium* disease-causing pathogens were developed using primers and or probes derived from RAPD fragments (Nicholson *et al.* 1996, 1998; Parry and Nicholson 1996; Schilling *et al.* 1996; Waalwijk *et al.* 2004) or low copy number functional genes (Glynn *et al.* 2005). The quantitative PCR assays described in this study had limits of detection of 0·0008 pg μl^{-1} (JBF) and 0·003 pg μl^{-1} (JBM). This represents between a five- and 50-fold greater sensitivity than previous competitive PCR assays for the individual species; (0·02 pg μl^{-1}) *F. culmorum* and *F. graminearum* (Nicholson *et al.* 1998) and (0·02 pg μl^{-1}) *M. nivale* (Nicholson *et al.* 1996) and (0·2 pg μl^{-1}) *M. majus* (Nicholson *et al.* 1996). Greater sensitivity is advantageous as it allows detection of low amounts of pathogen DNA in planta during initial stages of colonization. The greater sensitivity we observed likely stems from the high copy number of the target rDNA genes in fungal genomes in contrast to the likely low copy number of the RAPD fragments. The validation of the quantitative assays using three isolates of each species showed that there was no difference in the concentration of any species as determined by either assay.

The development and control of seedling blight of spring wheat caused by *F. graminearum sensu stricto* (syn. *F. graminearum* lineage 7) has been described previously (Jones 1999). As far as we know, no data are available on the epidemiology of *Fusarium* seedling blight of wheat caused by other lineages within the *F. graminearum* clade. Ear infections by separate species have been reported as optimal at 28–29°C for *F. graminearum*, 26·5°C for *F. culmorum* and 18°C for *Microdochium* sp. (Rossi *et al.* 2001). The mean maximum daily temperature during the mist irrigation process in our experiment was 18·2°C (minimum 13·2°C, maximum 24·4°C), which may explain the limited infection by *F. graminearum* and the higher amount of *Microdochium* quantified in seed.

In both experiments, those seed lots that contained the most *Microdochium* or *Fusarium* group DNA produced seedlings with the most *Microdochium* or *Fusarium* group DNA indicating effective transmission from seed to seedlings. Infection by the *Microdochium* seedling blight pathogens was more severe in untreated seedlings in experiment 2 than in experiment 1. For *Fusarium* seedling blight pathogens, however, infection in untreated seedlings was more severe in experiment 1 than experiment 2 despite more seed-borne *Fusarium* DNA in the experiment 2 seed. This could be a result of separate environmental conditions that favoured infection by each group of pathogens (Millar and Colhoun 1969; Parry *et al.* 1994). The mean daily soil temperature between drilling and sampling for experiment 1 was 12·2°C (minimum 8·0°C maximum 14·6°C) whereas for experiment 2 the mean soil temperature was 9·5°C (minimum 6·5°C maximum 11·7°C) between drilling and sampling. The lower soil temperature in experiment 2 may account for the higher level of infection by *Microdochium* compared with *Fusarium* pathogens. A further explanation may be that when severe disease caused by *Microdochium* seedling blight pathogens occur, infected seedlings may be more susceptible to infection by seed-borne *Fusarium*. Millar and Colhoun (1969) stated that when applied to the seed surface, high spore loads of *F. culmorum* could act as a substitute for unfavourable environmental conditions in determining seedling disease. Under favourable environmental conditions, however, *Microdochium* seedling blight pathogens can cause severe infection even if the number of spores per seed was extremely low (Colhoun 1970). The amount of *Fusarium* detected in the eight seed lots was much greater than *Microdochium* spp., in general, more *Microdochium* spp. than *Fusarium* was detected in infected seedlings except when *Fusarium* was present in the seed at the highest level. The conclusions of the earlier workers, based on the number of spores artificially applied to the surface of wheat seeds, may be corroborated by results from this study where the amount of pathogen DNA contained within a sample of seed has been quantified.

Beret Gold (a.i. fludioxonil) and Sibutol (a.i. bitertanol + fuberidazole) were chosen in this investigation as *in vitro*, they are the most active fungicide seed treatments against isolates of *M. nivale* and *M. majus* (Glynn 2002). We found that fludioxonil alone showed better performance than bitertanol + fuberidazole towards

Microdochium. This was significantly better when the pressure from infection was particularly high in experiment 2. The level of control for the *F. culmorum*-infected seed was high (>90%) for both fungicides. No significant benefit was apparent in our experiments for the commercial seed with low infection. This is not unexpected as working with treated and untreated seeds with low *Microdochium* infection, Paveley and Davies (1994) found no benefit from seed treatments when seed was sown at a number of field sites in the UK.

There are conflicting reports on the severity of infection of seedlings surviving pre- or post-emergence seedling blight death. Humphreys *et al.* (1995) reported that in field studies and in trays of compost, seedlings that survived pre-emergence mortality caused by *Microdochium* or *Fusarium* spp. were largely free from infection following establishment. Hare (1997) however, reported that all seedlings surviving pre-emergence death caused by seed-borne *Microdochium* spp. in experiments performed at 6°C exhibited stem-base disease symptoms. Severe symptoms were also reported on seedlings that survived pre- and post-emergence death caused by *F. graminearum* (Kane and Smiley 1987). In our experiments, treatments with the lowest emergence scores (data not shown) had higher pathogen DNA contents and are consistent with the findings of Hare (1997) and Kane and Smiley (1987). The contradiction to the findings of Humphreys *et al.* (1995) may be a result of the detection of both symptom-causing and symptom-less pathogen infections in our study. The seed treatments used in our experiments were effective in reducing pathogen infection, as measured by reduction in fungal DNA, during the early stages of plant development. Further experiments that relate the amount of pathogen DNA at the seedling stage to the amount present later in crop development and at harvest are warranted in order to determine the overall effectiveness of the treatments used.

The competitive PCR assays for seedling blight pathogens in the genera *Microdochium* and *Fusarium* described here present significant advantages over traditional methods as they are able to detect symptomless pathogen infection and assess the quantity of fungi present. The assays we describe are also more sensitive than previously described PCR-based methods of quantification and will prove useful for future studies on the epidemiology and control of *Fusarium* complex diseases of small grain cereals.

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References

Bateman, G.L., Kwasna, H. and Ward, E. (1996) Relationships among *Fusarium* spp estimated by comparing restriction fragment length polymorphisms in polymerase chain reaction-amplified nuclear rDNA. *Can J Microbiol* **42**, 1232–1240.

Beck, J.J. (1998) *Detection of Fungal Pathogens Using the Polymerase Chain Reaction*. US Patent 5 814 453.

Browne, R.A. and Cooke, B.M. (2005) Resistance of wheat to *Fusarium* spp. in an in vitro seed germination assay and preliminary investigations into the relationship to *Fusarium* head blight resistance. *Euphytica* **141**, 23–32.

Cockerell, V., Kenyon, D.M., Mulholland, V., Bates, J.A., McNeil, M., Law, J.R., Handy, C.L., Roberts, A.M.I. *et al.* (2004) *Cereal Seed Health and Seed Treatment Strategies: Exploiting New Seed Testing Technology to Optimise Seed Health Decisions for Wheat*. Home Grown Cereals Authority Project Report No. 340.

Colhoun, J. (1970) Epidemiology of seed borne *Fusarium* diseases of cereals. *Ann Acad Sci Fenn Ser* **168**, 31–36.

Colhoun, J., Taylor, G.S. and Tomlinson, R. (1968) *Fusarium* diseases of cereals II. Infection of seedlings by *Fusarium culmorum* and *Fusarium avenaceum* in relation to environmental factors. *Trans Br Mycol Soc* **51**, 397–404.

Cook, R.J. (1980) *Fusarium* foot rot of wheat and its control in the Pacific Northwest. *Plant Dis* **64**, 1061–1066.

Daamen, R.A., Langerak, C.J. and Stol, W. (1991) Surveys of cereal diseases and pests in the Netherlands III. *Monographella nivalis* and *Fusarium* spp. in winter wheat fields and seed lots. *Neth J Plant Pathol* **97**, 105–114.

Doohan, F.M., Parry, D.W. and Nicholson, P. (1999) *Fusarium* ear blight of wheat: the use of quantitative PCR and visual disease assessment in studies of disease control. *Plant Pathol* **48**, 209–217.

Doohan, F.M., Brennan, J. and Cooke, B.M. (2003) Influence of climatic factors on *Fusarium* species pathogenic to cereals. *Eur J Plant Pathol* **109**, 755–768.

Edwards, S.G., Pirgozliev, S.R., Hare, M.C. and Jenkinson, P. (2001) Quantification of trichothecene-producing *Fusarium* species in harvested grain by competitive PCR to determine the efficacy of fungicides against *Fusarium* head blight of winter wheat. *Appl Environ Microbiol* **67**, 1575–1580.

Fernandez, M.R. and Chen, Y. (2005) Pathogenicity of *Fusarium* species on different plant parts of spring wheat. *Plant Dis* **89**, 164–169.

Glynn, N.C. (2002) Studies on the epidemiology and chemical control of *Fusarium* seedling blight of wheat using molecular techniques. *PhD Thesis*, Harper Adams University College, Shropshire, UK.

Glynn, N.C., Hare, M.C., Parry, D.W. and Edwards, S.G. (2005) Phylogenetic analysis of EF-1 alpha gene sequences from isolates of *Microdochium nivale* leads to elevation of

varieties *majus* and *nivale* to species status. *Mycol Res* **109**, 872–880.

Hare, M.C. (1997) Epidemiology and control of *Fusarium* seedling blight of winter wheat (*Triticum aestivum* L.). *PhD Thesis*, Harper Adams University College, Shropshire, UK.

Hilton, A.J., Jenkinson, P., Hollins, T.W. and Parry, D.W. (1999) Relationship between cultivar height and severity of *Fusarium* ear blight in wheat. *Plant Pathol* **48**, 202–208.

Humphreys, J., Cooke, B.M. and Storey, T. (1995) Effects of seed-borne *Microdochium nivale* on establishment and grain yield of winter-sown wheat. *Plant Var Seeds* **8**, 107–117.

Jones, R.K. (1999) Seedling blight development and control in spring wheat damaged by *Fusarium graminearum* Group 2. *Plant Dis* **83**, 1013–1018.

Kane, R.T. and Smiley, R.W. (1987) Relative pathogenicity of selected *Fusarium* species and *Microdochium bolleyi* to winter wheat in New York. *Plant Dis* **71**, 177–181.

Lee, S.B. and Taylor, J.W. (1990) Isolation of DNA from fungal mycelia and single spores. In *PCR Protocols: A Guide to Methods and Applications* ed. Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. pp. 282–287. San Diego, CA: Academic Press.

Millar, C.S. and Colhoun, J. (1969) *Fusarium* diseases of cereals VI. Epidemiology of *Fusarium nivale* on wheat. *Trans Br Mycol Soc* **52**, 195–204.

Nicholson, P., Lees, A.K., Maurin, N., Parry, D.W. and Rezanoor, H.N. (1996) Development of a PCR assay to identify and quantify *Microdochium nivale* var. *nivale* and *Microdochium nivale* var. *majus* in wheat. *Physiol Mol Plant Pathol* **48**, 257–271.

Nicholson, P., Simpson, D.R., Weston, G., Rezanoor, H.N., Lees, A.K., Parry, D.W. and Joyce, D. (1998) Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. *Physiol Mol Plant Pathol* **53**, 17–37.

Nicholson, P., Turner, A.S., Edwards, S.G., Bateman, G.L., Morgan, L.W., Parry, D.W., Marshall, J. and Nuttall, M. (2002) Development of stem-base pathogens on different cultivars of winter wheat determined by quantitative PCR. *Eur J Plant Pathol* **108**, 163–177.

Parry, D.W. and Nicholson, P. (1996) Development of a PCR assay to detect *F. poae* in wheat. *Plant Pathol* **45**, 383–391.

Parry, D.W., Pettitt, T.R., Jenkinson, P. and Lees, A.K. (1994) The cereal *Fusarium* complex. In *Ecology of Plant Pathogens* ed. Blakeman, P. and Williamson, B. pp. 301–320. Wallingford: CAB International.

Parry, D.W., Jenkinson, P. and McLeod, L. (1995) *Fusarium* ear blight in small grain cereals – a review. *Plant Pathol* **44**, 207–238.

Paveley, N.D. and Davies, J.M.L. (1994) Cereal seed treatment – risks, costs and benefits. In *Seed Treatment: Progress and Prospects, British Crop Protection Council Monograph No. 57* ed. Martin, T. pp. 27–35. Farnham: British Crop Protection Council Publication.

Pettitt, T.R., Parry, D.W. and Polley, R.W. (1993) Improved estimation of the incidence of *Microdochium nivale* in winter-wheat stems in England and Wales, during 1992 by use of benomyl agar. *Mycol Res* **97**, 1172–1174.

Rossi, V., Ravanetti, A., Pattori, E. and Giosue, S. (2001) Influence of temperature and humidity on the infection of wheat spikes by some fungi causing *Fusarium* head blight. *J Plant Pathol* **83**, 189–198.

Schilling, A.G., Moller, E.M. and Geiger, H.H. (1996) Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. *Phytopathology* **36**, 515–522.

Simpson, D.R., Weston, G.E., Turner, J.A., Jennings, P. and Nicholson, P. (2001) Differential control of head blight pathogens of wheat by fungicides and consequences for mycotoxin contamination of grain. *Eur J Plant Pathol* **107**, 421–431.

Waalwijk, C., de Koning, J.R.A., Baayen, R.P. and Gams, W. (1996) Discordant groupings of *Fusarium* spp. from sections *Elegans*, *Liseola* and *Dlaminia* based on ribosomal ITS1 and ITS2 sequences. *Mycologia* **88**, 361–368.

Waalwijk, C., Kastelein, P., Vries, I., Kerenyi, Z., Lee, T., Hesselink, T., Kohl, J. and Kema, G. (2003) Major changes in *Fusarium* spp. in wheat in the Netherlands. *Eur J Plant Pathol* **109**, 743–754.

Waalwijk, C., van de Heide, R., de Vries, I., van der Lee, T., Schoen, C., Corainville, G.C-d., Häuser-Hahn, I., Kastelein, P. *et al.* (2004) Quantitative detection of *Fusarium* species in wheat using TaqMan. *Eur J Plant Pathol* **110**, 481–494.

White, T.J., Bruns, S., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications* ed. Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. pp. 315–322. San Diego, CA: Academic Press.

Wiese, M.V. (1987) *Compendium of Wheat Diseases*. St Paul, MN: American Phytopathological Society.

Zadoks, J.C., Chang, T.T. and Konzak, C.F. (1974) A decimal code for the growth stages of cereals. *Weed Res* **14**, 415–421.